Effect of Bovine Free Martin Serum on *in vitro* Development of Bovine Embryos Derived from *in vitro* Maturation and Fertilization

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The media used for *in vitro* maturation and the culture of bovine *in vitro*-fertilized oocytes are usually supplemented with serum collected from animals²⁰⁾. Requirements for the development of early mammalian embryos of domestic animals have not been defined¹⁶⁾. But serum may also contain components beneficial to bovine embryo development. The common serum preparations used are fetal calf serum (FCS), new born calf serum (NCS), estrous bovine serum (ES) and bovine serum albumin (BSA)^{10, 15, 21)}. However, free martin serum (FS) has never been used. Serum may enhance embryo development by providing certain growth factors, and we confirmed that the concentration of insulin like growth factor I (IGF-I) in FS (heat-inactivated) was higher than in

other sera (18.2 ng/ml).

In this study, we attempted to evaluate the effects of free martin serum and other sera in TCM 199 for their ability to support the development of bovine embryos. We also tried to confirm the effects of IGF-I which showed a higher concentration in FS, as mentioned above.

Materials and Methods

In vitro maturation (IVM) : Ovaries were collected from cows and heifers at a local slaughterhouse ; maintained at 20–25°C and transported to the laboratory within 2 hours after slaughter. Cumulus-oocyte complexes were aspirated from small antral follicles of 2 to 8 mm in diameter with a 21 G needle attached to

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a 10 ml disposable syringe. Oocytes were washed three times with tissue culture medium TCM-199 (GIBCO, NY, USA) supplemented with penicillin-streptomycin (10 μ l/ml: penicillin G, 10000 U/ml; streptomycin, 10000 μ g/ml; GIBCO, NY, USA) and 10% (v/v) calf serum (CS; GIBCO, NY, USA). Oocytes with intact cumulus cells and an evenly granulated cytoplasm were cultured in microdrops (100 μ l) of TCM-199 supplemented with penicillin-streptomycin and heat-inactivated CS. Oocytes were cultured for 22 hours in a CO₂ incubator maintained at 38.5°C under 5% CO₂ in air and 95% humidity.

In vitro fertilization (IVF): *In vitro* fertilization was carried out using the procedures reported by TAKADA *et al.*¹⁷⁾. Briefly, 0.5 ml plastic straws of frozen Japanese Black sire semen were thawed in a waterbath at 37°C and then washed twice with BRACKETT and OLIPHANT²⁾ medium without bovine serum albumin, but supplemented with 10 mM caffeine, by centrifugation at 400 g for 5 min. The final pellet was resuspended with the medium to adjust to a concentration of 2.0×10^7 spermatozoa per milliliter. An equal volume of BRACKETT and OLIPHANT medium supplemented with 10 mg/ml BSA (SIGMA, USA) and 10 I.U./ml heparin (NOVO-KODAMA, JAPAN) was added to the sperm suspension. Heparin-treated spermatozoa were placed in a 100 μ l microdrop covered with mineral oil and the cumulus-oocyte complexes were placed in the microdrop.

In vitro culture: After six hours incubation with spermatozoa, cumulus-oocyte complexes were washed with a development medium (TCM199 supplemented with 10% ES) to remove excess spermatozoa.

Treatment: Seventy two hours after insemination (day 3), cumulus cells surrounding embryos were removed by pipetting and the developmental stage of the embryos was examined. In the experiment, embryos that developed into four to seven and eight-cells

were randomly allocated to four treatments media: TCM 199 supplemented with 10% calf serum, 10% fetal calf serum, 10% free martin serum, 10% estrous cow serum and 10% calf serum supplemented with 50 ng/ml IGF-1 (rIGF-1; TOYOBO, JAPAN). Free martin serum was collected from three different free martin cattle ranging from 3-24 months and estrous cow serum was collected from three cows in estrus.

The total cell numbers of blastocysts in CS and FS were counted via the methods of PURSEL *et al.*¹²⁾.

Statistical Analysis: The chi-square test was used to test the differences in the developmental rate to the blastocyst among the groups.

Results

The percentage of four to seven-cell embryos that developed to the blastocyst stage in ES, FS, FCS and CS were 19%, 44%, 12% and 9%, respectively. Development of eight-cell embryos to the blastocyst stage in ES, FS, FCS and CS was 32%, 44%, 11% and 20% respectively (Table 1). The highest proportion of embryos that developed to the blastocyst stage was observed in the FS treatment. There were significant differences in the development among the groups. Furthermore, increased rate of blastocyst formation was observed in FS-TCM199 compared with other media. The total cell numbers of blastocysts in CS and FS were 97.0 ± 10.6 , and 113.3 ± 9.4 . There were no significant differences among the treatments.

Discussion

Many reports exist on bovine oocytes matured, fertilized and cultured *in vitro*. Although the rates of *in vitro* maturation and fertilization are high, the developmental rate of embryos cultured *in vitro* is still low.

Many workers have investigated whether or not hormones and sera are necessary for *in vitro* maturation. The presence of serum in

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Table 1. *In vitro* development of bovine embryos to the blastocyst stage (day 8)¹ in different sera

Embryo (day 3) ¹	Estrous serum	Treatments			Fetal calf serum	Calf serum	Calf serum + IGF-1
		Free martin serum					
		A	B	C			
4~7 cells	19.1% ^a (17/89) ²	51.5% ^b (84/163)	34.7% ^c (35/101)	40.3% ^{bc} (25/62)	12.9% ^a (11/85)	9.2% ^a (6/65)	— —
8~ cells	32.2% ^{abd} (51/158)	39.0% ^{bcd} (16/41)	45.0% ^c (54/120)	41.5% ^{cd} (32/77)	11.1% ^{ac} (3/27)	20.0% ^{ae} (10/50)	51.4% ^{cd} (104/201)

¹ insemination = day 0.

² No. of embryos to the blastocyst stage (day 8)/No. of treated embryos

* Different superscripts are significantly different (P<0.05)

the culture medium was necessary for oocyte maturation, fertilization and development, since sera could provide cumulus-oocyte complexes with beneficial components for *in vitro* maturation^{9,18,19}. Serum obtained from estrous cows have been used to improve *in vitro* maturation and fertilization rates^{20,21}.

In the present study, the medium supplemented with FCS and CS effectively supported the development of bovine oocytes with cumulus cells. In our study, without any co-culture, free martin serum-supplemented TCM 199 resulted in a higher incidence of developed blastocysts than the other serum-supplemented TCM 199. This study has found that four-to-eight cell bovine embryos can develop into blastocysts (without a co-culture) in a culture medium containing free martin serum. Free martin serum might provide beneficial components, such as hormones, growth factors, proteins, amino acid and some as yet undefined factors. In this study, we found that the insulin like growth factor-I level in free martin serum was high compared with heat-activated other serum. We suspect that IGF-I supported embryonic development, because the addition of IGF-I to the *in vitro* culture media can slightly increase the developmental rates of embryos and the number of blastocyst nuclei. HARVEY and KAYE observed that in an embryo exposed to IGF-I, protein synthesis increased, and they showed that IGF-I could

interact with the insulin receptor making it unclear whether the observed affects were mediated by insulin or IGF-I receptor^{5,6}. RECHLER and NISSLEY reported that IGF-I could react with insulin receptors¹³. KADOWAKI *et al.* suggested that an insulin receptor could mediate the effects of IGF-I and insulin⁸. Growth factors play an important role in embryonic development⁷.

It has been reported that bovine blastocysts developed *in vivo* had about 100 to 160 blastomeres at the blastocyst stage^{3,9}. The present study resulted in lower numbers of nuclei compared with embryos produced *in vivo*.

The co-culture of *in vitro*-fertilized oocytes with cumulus cells provides an appropriate environment for bovine embryonic development^{1,4,11,12,14,16,19}. These results suggest that co-culture systems not only support embryonic development but also improve the quality of embryos produced *in vitro*.

In conclusion, free martin serum added to the culture medium improves the developmental capacity of bovine embryos *in vitro* without any co-culture. Free martin serum is a rich source of IGF-I. And perhaps, undetected components in serum would perform well in supporting embryonic development, but this no co-culture system can not improve the quality of embryos.

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